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Cancer cells have long been known to express glycosylation patterns that are different from those found on normal cells from the same tissue. Many tumor-specific carbohydrate antigens possess the monosaccharide sialic acid, and as a consequence, many tumor cells express high levels of sialic acid compared to normal cells. Thus, any agent that targets sialic acid specifically could be used for tumor targeting. Preliminary work on this project has shown that unnatural sialic acids can be introduced onto tumor cells metabolically by feeding the cells unnatural analogs of their biosynthetic precursors. The unnatural analogs can include reactive functional groups capable of covalent reaction with exogenous probes. For example, an azide-functionalized analog of Nacetylmannosamine is converted by tumor cells to the corresponding sialic acid, and the azide can be covalently reacted on the cell surface with triarylphosphine probes in vivo. The objective of this project is to develop a novel breast cancer targeting method that exploits the selective chemical reactivity of unnatural sialic acids for delivery of imaging reagents. The immediate application is a new method for non-invasive detection and diagnosis of cancer. Longer-term applications include targeted anti-cancer drugs and vaccines.

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Introduction

Breast cancer is the leading cause of cancer death for women worldwide (1). While a cure for this malignancy remains elusive, early diagnosis of breast cancer correlates with both improved therapeutic outcomes and increased survival rates for patients (2-4). Traditional medical procedures used to detect breast cancer (i.e., mammography, ultrasound, and MRI), rely on gross anatomical differences between malignant and healthy tissues, leaving many tumors undetected or misdiagnosed (4). Therefore, much effort is now directed toward developing alternative breast cancer detection technologies that exploit the differences between cancer and normal cells at the *molecular* level (2, 5-14).

One striking difference between cancerous and normal tissue lies in the composition of their cell-surface carbohydrate structures. Breast cancer cells have long been known to display glycosylation patterns that are different from those found on normal cells from the same tissue (15, 16). Many cancer-associated carbohydrate epitopes possess the monosaccharide sialic acid, and as a consequence, the cancer cells tend to express high levels of sialic acid compared to their normal counterparts. Overexpression of sialic acid has been correlated with the malignant and metastatic phenotypes in human breast cancers (15-18). Thus, an imaging strategy that targets sialic acid could potentially be used for breast tumor diagnostics.

The goal of this proposal is to develop a non-invasive breast cancer imaging strategy that exploits the high levels of sialic acid on tumor cells. In previous work, we have shown that *unnatural* sialic acids can be introduced onto cultured cells metabolically by feeding the cells analogs of their biosynthetic precursors (19, 20). The unnatural analogs can be endowed with bioorthogonal functional groups capable of covalent reaction with exogenous probes. For example, an azide-functionalized analog of *N*-acetylmannosamine termed ManNAz is converted by cells to the corresponding sialic acid (SiaNAz). The sialic acid-associated azides can be covalently tagged using triarylphosphine probes, a reaction termed the Staudinger ligation, without any apparent toxic effects. The objective of this project is to employ unnatural sialic acid biosynthesis and the Staudinger ligation to target diagnostic imaging reagents to breast cancer tissue. This would require that the Staudinger ligation perform in living animals, an unprecedented event for a covalent reaction. Thus, a major goal of the project is to explore the metabolism of azido sugars in animal model, and to evaluate the Staudinger ligation as a means for in vivo targeting. A second goal of the project was to synthesize phosphine probes for non-invasive imaging. The accomplishments relevant to these goals are summarized below.

Body

In vivo Staudinger ligation

During the granting period, we demonstrated that the Staudinger ligation can indeed be used to target azido sialic acids in living animals (21). We first demonstrated that *N*-azidoacetylmannosamine (ManNAz) is metabolized to the corresponding unnatural sialic acid (SiaNAz, Fig. 1B) in murine splenocytes (Fig. 2). Acetylated ManNAz in an aqueous DMSO solution was injected into the peritoneal cavity of mice at various daily doses for seven days. On the eighth day, the mice were sacrificed and their splenocytes harvested. The cells were reacted with a phosphine-FLAG peptide probe (Fig. 2) then analyzed by flow cytometry. As shown in Fig. 3, splenocytes were labeled with the phosphine probe in a manner dependent on the dose of azido sugar. Splenocytes from mice that had not been injected with acetylated ManNAz did not undergo Staudinger ligation with phosphine-FLAG *ex vivo*.

We first performed these experiments in the serum esterase-deficient mouse strain $Es1^e/Es1^e$ (Fig. 3A and B) to avoid possible deacetylation of the substrate prior to cellular uptake. The importance of acetylation for efficient metabolism of ManNAz *in vivo* was confirmed by comparison of SiaNAz levels on splenocytes of wild-type mice (B6) treated with either the acetylated or free sugar (Fig. 3C). However, wild-type mice were later shown to metabolize acetylated ManNAz to SiaNAz at similar levels as the esterase-deficient mice,

suggesting that cellular uptake is faster than deacetylation in serum (Fig. 3D). This result bodes well for applications of unnatural sugar metabolism in a wide variety of mouse strains with normal serum esterase levels. In addition to the spleen, we observed SiaNAz-modified glycoproteins in the heart, liver, kidney and serum by reaction with phosphine-FLAG *ex vivo* followed by Western blot analysis (not shown).

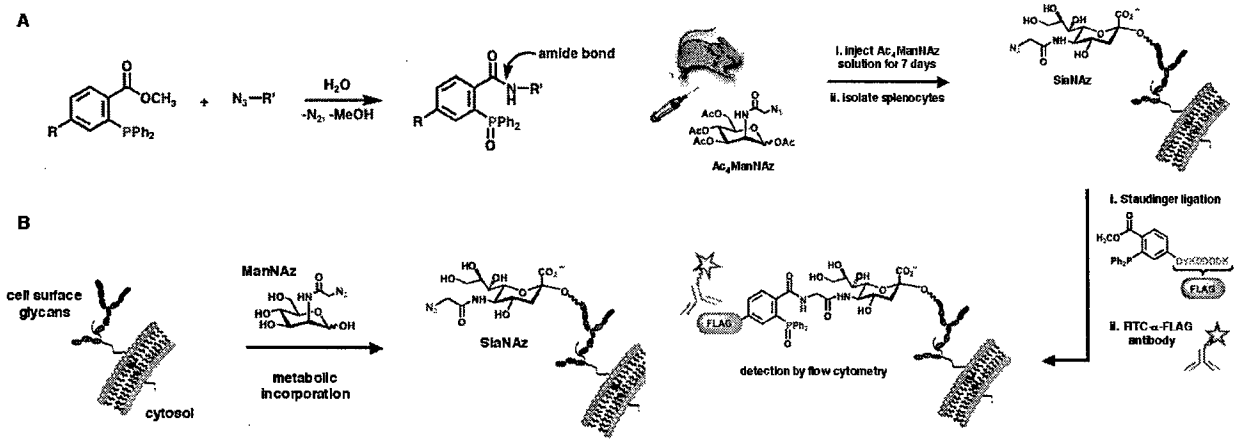

Figure 1 . **A**. Staudinger ligation. **B**. Conversion of ManNAz to cell surface SiaNAz.

Figure 2. Analysis of acetylated ManNAz metabolism *in vivo* .

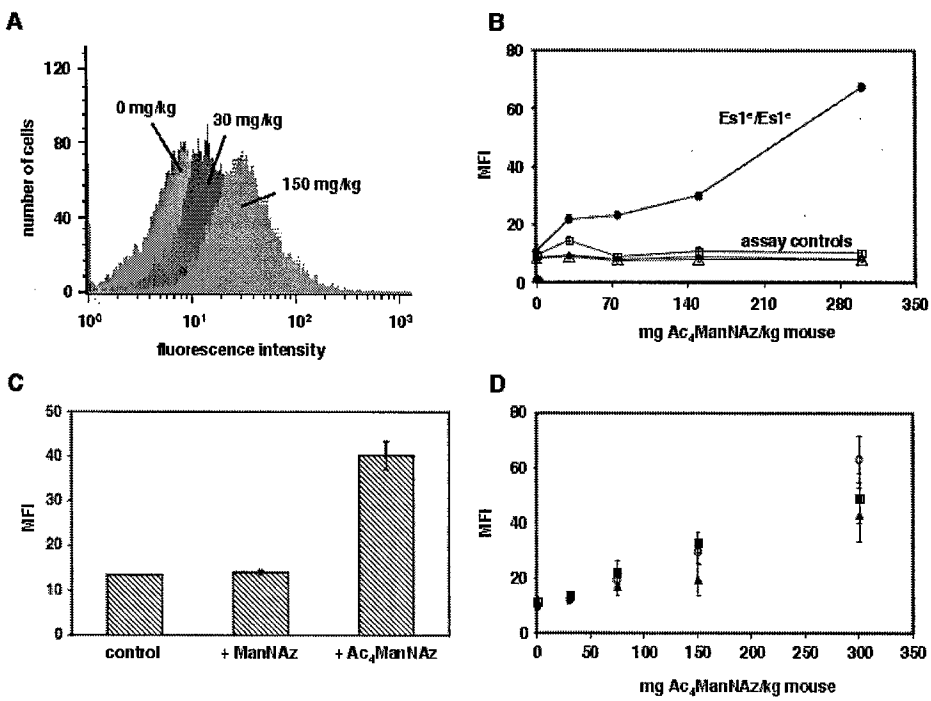

Figure 3. A. Flow cytometry analysis of splenocytes from $Es1^e/Es1^e$ mice injected with various doses of acetylated ManNAz. The cells were treated with phosphine-FLAG (250 μM) followed by a FITC-labeled anti-FLAG mAb, and analyzed by flow cytometry. **B.** Mean fluorescence intensity (MFI) of the cells from **A** as a function of azido sugar dose (●). Assay controls included unlabeled splenocytes from azido sugar-treated mice (Δ), the same splenocytes treated with phosphine-FLAG and a class-matched control mAb (○), and the same splenocytes treated anti-FLAG mAb only without prior Staudinger ligation with phosphine-FLAG (□). **C.** Splenocyte SiaNAz levels from wild-type mice treated with acetylated or free ManNAz **D.** SiaNAz levels on splenocytes from wild-type (○ and ■) and $Es1^e/Es1^e$ (▲) mice treated with various doses of acetylated ManNAz.

Having demonstrated that acetylated ManNAz is converted to SiaNAz in murine organs, we next investigated whether the Staudinger ligation could perform *in vivo*, thereby remodeling cells with novel epitopes in the context of a living animal. Mice were injected with the acetylated ManNAz for seven days (300 mg/kg), then injected with phosphine-FLAG (20 μmoles) on the eighth day. After 1.5 hours, the mice were sacrificed and their splenocytes were analyzed for the presence of the FLAG epitope by flow cytometry. As shown in Fig. 4, only those mice that had been administered both the azido sugar and phosphine probe were positive for the FLAG neo-antigen.

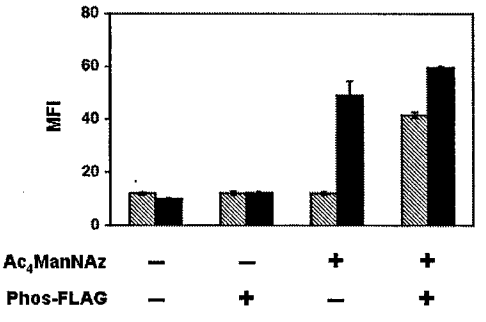

Figure 4. *In vivo* Staudinger ligation of splenocyte-associated SiaNAz with phosphine-FLAG. Grey – anti-FLAG mAb staining of cells directly harvested from mice. Black – anti-FLAG mAb staining of cells harvested from mice and then subjected to additional phosphine-FLAG treatment (250 μM) *ex vivo*.

Probes for noninvasive imaging

A fundamental goal in the field of molecular imaging is the identification of tissue-specific markers that can be targeted with probes for image contrast. To date, the field has focused on protein-based markers that are targeted with antibody conjugates, receptor ligands or enzyme substrates/inhibitors. Despite numerous reports of changes in cell-surface glycosylation associated with disease, to our knowledge there are no reports of glycan-specific imaging. The ability to chemically modify cell surface glycans in living animals provides a means to monitor changes in glycosylation in a physiologically authentic context. For example, changes in glycan profiles that occur during tumor growth and metastasis could be visualized in real time.

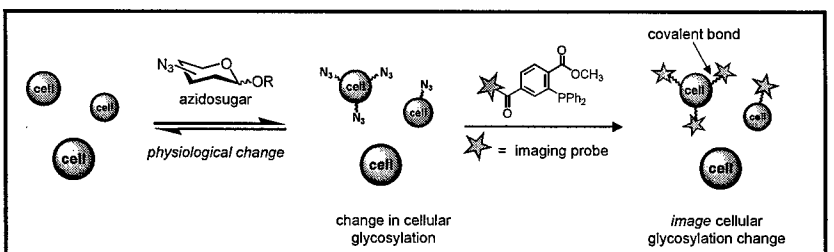

Figure 5. Proposed strategy for imaging changes in glycosylation in vivo. Physiological changes such as malignant transformation may be reflected in altered cell surface glycosylation. Such changes may be visualized by metabolic oligosaccharide engineering and subsequent

The proposed strategy, depicted schematically in Fig. 5, has several advantages over other conceivable approaches to glycan visualization: (1) The azidosugar and phosphine imaging probe are small molecules with the potential for broad tissue penetrance. By contrast, antibody conjugates have limited tissue access. (2) The targeting event involves a covalent bond at the cell surface rather than non-covalent association. Unbound imaging probes can be cleared without rapid loss of specific signal. This is key to achieving high signal-to-noise for imaging modalities with high sensitivity. (3) The approach permits imaging of metabolic flux, a parameter that cannot be monitored by conventional glycan-binding reagents.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are used extensively for detecting cancer and for noninvasive monitoring of biochemical changes in living subjects. For these imaging modalities, a radionuclide (a β-emitting isotope and a γ-emitting isotope for PET and SPECT, respectively) is administered to a patient and used to generate images depicting the concentration/location of the tracer. Current PET and SPECT methods for cancer detection achieve image contrast by either (1) accumulation of the unnatural sugar [^{18}F]-2-fluoro-2-deoxy-glucose (FDG) in tumor tissue relative to healthy tissue owing to enhanced metabolism in tumor cells, or (2) binding of radiolabeled probes (i.e., antibodies and

receptor ligands) to molecules overexpressed on tumor cell surfaces. These approaches are not without limitations. FDG is not specific for malignant cells, as all cells take up FDG to some extent. Additionally, antibody-based imaging probes are limited by their long serum half-lives (several weeks), which creates high background signal outside of the tumor environment for an extended period of time. Therefore, identifying alternative tumor cell markers that can be selectively targeted with radionuclide probes would benefit the field of PET/SPECT imaging in cancer diagnostics.

Metabolic oligosaccharide engineering with ManNAz and GalNAz may provide a means to monitor changes in glycosylation associated with malignancy using PET and SPECT. Therefore, our first completed synthetic targets were phosphine-based probes for SPECT and PET imaging of azidosugar metabolism. Our initial radionuclide targets were ^{125}I- and ^{18}F-radiolabeled Phos-FLAG analogs **7** and **8** (Fig. 6), designed for SPECT and PET imaging, respectively. While both imaging modalities can be used to monitor biological changes in cancer cells with high sensitivity, each technology has its own distinct advantages. PET imaging is 1-2 orders of magnitude more sensitive than SPECT owing to its use of beta-emitting isotopes (such as ^{18}F), but SPECT can be performed with several readily available gamma-ray emitting isotopes (such as ^{125}I) that possess relatively long half-lives.

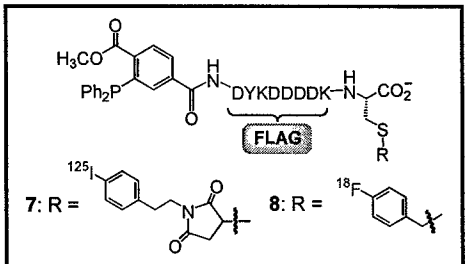

Figure 6. Radionuclide reagents for noninvasive imaging.

Both **7** and **8** were prepared from a derivative of Phos-FLAG bearing a C-terminal cysteine residue. This allowed convergent coupling with the known radiolabeled compounds [^{18}F]-*p*-fluorobenzyl iodide and [^{125}I]-*p*-iodophenethylamine maleimide, respectively. The radionuclides are presently being used in imaging experiments with our collaborator Martin Pomper at the Johns Hopkins Molecular Imaging Center. Importantly, compounds **7** and **8** satifsy several requirements of molecular imaging probes; namely, the isotopes are far removed from the phosphine center so as not to perturb the reactivity of the probe, and the molecules are water-soluble. Additionally, these hydrophilic imaging agents possess relatively low molecular weights and should be rapidly cleared from non-target areas in living animals, thereby reducing background signal. Finally, the FLAG epitope provides an independent means of probe detection for correlations with imaging data (vide infra).

Additionally, we prepared phosphine probes bearing chelators for radioactive metals: CDTA (in **9**) for use with the gamma-ray emitting isotope ^{111}In, and DOTA (in **10**) for use with both ^{111}In and ^{64}Cu, a PET radionuclide (Fig. 7). The syntheses of compounds **9** and **10** were straightforward, as the free amines of the chelators are commercially available and were readily coupled with the phosphine carboxylic acid, similar to the synthesis of Phos-FLAG [21]. Prior to in vivo work, we will confirm that the probes undergo the Staudinger ligation in model reactions and on cultured cells.

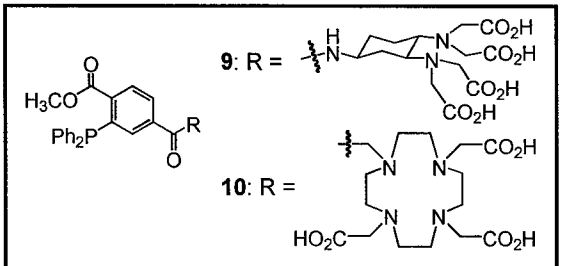

Figure 7. Metal-chelating scaffolds for noninvasive imaging.

Interest in fluorescence imaging is rapidly increasing due to its high sensitivity (detections limits in the 10^{-12} M range), recent improvements in instrumentation, and relatively low cost (no cyclotron or superconducting magnet required). Fluorescent dyes can be designed with a wide range of spectral properties and multiple dyes can be imaged simultaneously and independently. The initial targets will be phosphine-rhodamine conjugates (represented by **11** in Fig. 8). The rhodamine scaffold has a λ_{ex} = 567 nm and λ_{em} = 589 nm, wavelengths with adequate tissue penetrance for imaging subcutaneous tumors. We synthesized the dye on a multigram scale using standard amide coupling procedures. We plan to

test a variety of linkers, including the FLAG peptide, in order to optimize metabolic stability and pharmacokinetic properties. In addition, we will explore near-infrared (NIR) dyes that excite and emit at longer wavelengths with superior tissue penetrance. However, these dyes are expensive and difficult to synthesize. Thus, we intend to optimize the system using rhodamine-based dyes before applying NIR dyes.

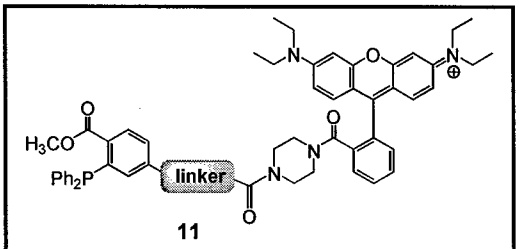

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Figure 8. Phosphine-rhodamine conjugate for fluorescence imaging.

Key Research Accomplishments

- The Staudinger ligation can be used to target murine tissues with reagents on the basis of glycosylation patterns.

- Synthesis of phosphine-based imaging reagents for PET, SPECT, MRI and fluorescence imaging

Reportable Outcomes

Conference abstracts:

Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Probing Azidosugar Metabolism in vivo Using the Staudinger Ligation.* National Meeting of the American Chemical Society, New York, NY (Sep 2003)

Staudinger Ligation. Annual Conference of the Society for Glycobiology, San Diego, CA (Dec 2003)

Hang, H. C.; Bertozzi, C. R. *A Chemical Approach Towards Mucin-type O-linked Glycoproteomics Using the Staudinger Ligation.* Annual Conference of the Society for Glycobiology, San Diego, CA (Dec 2003)

Prescher, J. A.; Dube, D. H.; Bertozzi, C. R. *Chemically Remodeling Cell Surfaces in Living Animals.* Society for Glycobiology Meeting, San Diego, CA (Dec 2003)

Prescher, J. A. *Chemical Approaches to Studying Protein Glycosylation* International Symposium on Recent Advances in the Chemistry and Biomedical Aspects of Complex Carbohydrates. Alberta, Canada (Apr 2004)

Bertozzi, C. R.
Invited Lectures:

Annual Conference of the Society for Glycobiology, San Diego, CA	Dec 2003
Center for Synthesis and Chemical Biology Symposium, "Recent Advances in Synthesis and Chemical Biology II", Dublin, Ireland	Dec 2003
UCSF Prostate Cancer SPORE Retreat, South San Francisco, CA	Jan 2004
Chemistry and Biology: Partners in Decoding the Genome, National Institutes of Health, Bethesda, MD	Mar 2004
Symposium on Chemistry as a Life Science XII, Rutgers, Newark, NJ	Mar 2004
30th Reaction Mechanisms Conference, Northwestern Univ., IL	Jun 2004

Conclusions

Our work during the granting period has established the Staudinger ligation as the first organic transformation that can specifically remodel cells in the context of living organisms. This discovery sets the stage for targeting of imaging agents to cancer cells for non-invasive diagnostic applications. We have also prepared a series of imaging agents that are now being used in vivo. Our work in the next months will determine their selectivity for tumor vs normal tissues. The imaging agents synthesized during this granting period may represent a new class of diagnostic probes for early detection of breast cancer based on differential glycosylation. The next phase of this research is to evaluate their ability to target cells expressing azido sugars in vivo, on normal and cancer tissues.

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